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PETROLEUM DEGRADATION IN LOW TEMPERATURE MARINE AND ESTUARINE E--ETC(U)
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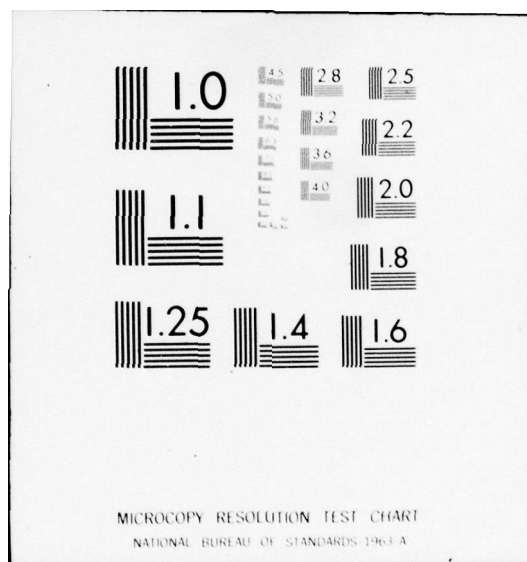
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Contract N00014-76-C-0138

Task No. NR 205-010

FINAL REPORT

Petroleum Degradation in Low Temperature
Marine and Estuarine Environments

by

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1 January 1978



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20. Abstract

△ Hydrocarbon utilizing bacteria were isolated from several low temperature water and sediments and were found to represent 15 different genera. All isolated were psychrotolerant and had Q10 values ranging from 1.3-2.4 and metabolized representative aromatic, naphthenic and aliphatic hydrocarbons. In situ degradation rates were demonstrated to be much lower than laboratory rates and were on the order of magnitude of ngs of substrate per day. The sediment microbial populations in an oiled beach were shown to select for hydrocarbon metabolizing organisms and this selection correlated with degradation of hydrocarbons in the sediments. Hydrocarbon metabolizing microorganisms contain inclusions not present in the same organisms grown on peptone. The inclusions are identified as hydrocarbon oxidation products and are not pooled hydrocarbon. △

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A. Objectives:

The stated objectives of this project were:

- (1) The isolation of microorganisms which metabolize hydrocarbon from low temperature marine and estuarine environments,
- (2) To investigate the types of hydrocarbons metabolized by isolates,
- (3) To compare rates of hydrocarbon degradation at psychrophilic and mesophilic temperatures,
- (4) To investigate the ultrastructure of hydrocarbon degrading microorganisms, and
- (5) To apply these techniques to a study of the changes in the microbial flora and chemical analyses in Gaspee Point sediments following a No. 6 Fuel Oil Spill.

B. Methodology:

The methods used in this project are described in detail in each of the preceding annual reports.

C. Results:

1. Isolates and substrate utilization

A total of 79 different organisms were isolated by enrichment culture from various locations in Narragansett Bay. Enrichments were performed at ambient temperatures of 8°C and 16°C using the hydrocarbon substrates, naphthalene, kerosene, Nos. 1, 2, 4 and 6 fuel oils as sole sources of carbon for growth. Similar enrichments were performed on sediments from Chedabucto Bay in Nova Scotia at 25°C, 1°C and 0°C to yield 47 isolates. Cape Simpson, Alaska sediments enriched at 16°C yielded an additional 14 isolates.

The majority of the isolates were classified to genus level which identified 15 different genera able to metabolize one or more pure n-alkanes, cyclic aliphatic and/or aromatic hydrocarbons. Hydrocarbon utilization profiles were determined on selected isolates representative of the various genera. Some of the isolates were able to metabolize only aliphatic hydrocarbons, whereas many isolates were able to metabolize aromatic, cyclic and n-alkane substrates. In all cases isolates able to metabolize aromatic hydrocarbon also utilized n-alkanes.

2. Temperature effects on growth rate and degradation rate

A study of the growth temperatures of the isolates on hydrocarbons demonstrated that they had a broad range of growth temperatures (0°C-24°C) and should be classed as psychrotolerant rather than psychrophilic bacteria. (Table 1).

Table 1

Ratio of selected Narragansett Bay isolates growing on various hydrocarbon substrates after 14 days incubation at different temperatures

Substrate	Ratio of Isolates Growing			
	0C	8C	16C	24C
n-hexadecane	15/24	24/24	24/24	22/34
methylcyclohexane	9/24	16/24	13/24	13/24
n-hexylbenzene	11/24	11/24	9/24	13/24
naphthalene	6/24	10/24	11/24	12/24

Growth studies with selected isolates were performed in seawater-salts medium containing n-hexadecane, naphthalene or other hydrocarbons as the carbon source at 10C and at 20C. The significant effect of temperature was the increased lag time of the cultures grown at 10C. During the exponential growth phase generation times (G) were calculated from viable plate count data and demonstrated Q_{10} values ranging from 1.6 to 2.2. The demonstration of Q_{10} values in this range were not in agreement with the suggestion of Zo Bell (1969) that there would be a two to threefold increase in G for every 10C reduction in temperature. The increased lag phase was similar to the response reported by Atlas and Bartha (1972) which they attributed to the presence of volatile toxic components in Sweden crude oil used as the growth substrate. The observations with pure n-hexadecane suggests that lag effects need not be related to substrate toxicity.

The details of the isolates, their substrate responses, temperature effects and isolate descriptions are reported in a series of reports and publications (Traxler, 1974; Cundell and Traxler, 1973; Cundell and Traxler, 1974; Cundell and Traxler, 1976).

A review of the literature on hydrocarbon degradation rates in ocean environments clearly demonstrated that comparisons of the values obtained by various investigators are not feasible because of variations in hydrocarbon substrates, bacterial inocula, cultural conditions and methods of measurement. It was apparent, however, that degradation rates from laboratory and simulated field conditions were considerably higher than actual *in situ* rates. The range of laboratory rates reported in the literature was from 0.33ug/cm³/day to 1.5mg/cm³/day.

Calculations from the persistence and degradation study in Narragansett Bay demonstrated a theoretical rate for dodecane utilization of 0.7ug of dodecane/gram of wet sediment/day whereas actual chemical analysis of sediments (4C) showed a rate of degradation of total alkanes which was 0.5 ug/gram dry sediment/day. Experiments using the modified BOD method with sediment and seawater as the inoculum showed an oxygen depletion rate of 2lug of oxygen per ml per day. The theoretical oxygen demands of

n-dodecane is 3.46 mg for its complete conversion to carbon dioxide and water. The degradation rate would therefore be 6.1 ug of hydrocarbon as dodecane per ml per day in this test system.

Bacterial isolated grown in pure culture will degrade hydrocarbons more rapidly than natural in situ populations. The availability of adequate phosphorous and nitrogen, good dispersion of the hydrocarbons, constant temperature and high inocula all favor an optimal degradation rate under laboratory conditions. We used laboratory degradation rates as comparative data to determine the effect of one variable such as temperature and as such we did not relate these rates directly to in situ environments.

Three isolates were used to determine the rate of dodecane utilization at 10C and 20C (Table 2). The average rate of degradation was expressed as the amount of hydrocarbon degraded per ml of culture per day as measured by gas chromatography.

Table 2

The effect of temperature on the degradation rate of n-dodecane by three isolates

Isolate	Degradation rate ug/ml/day		Q ₁₀
	20C	10C	
6-A-7-24-1	94	62	1.5
N-1-8-1	81	52	1.6
N-BP-8-71	84	46	1.8

This data confirms that the Q₁₀ may be considerably less than 2.0 which tends to minimize the effect of lower temperature on degradation rate. Williams' (1969) model for metabolic rates superimposed on variations in the annual temperature cycle indicates that 14C data would represent the average annual degradation rate for in situ measurements in Narragansett Bay. BOD measurements at 14C on Wilmar crude by Narragansett Bay seawater inocula provided an oxygen depletion rate of 1.25 mg O₂/liter/day. Using the theoretical oxygen demand for n-dodecane this calculates to an average degradation rate of 15 ng of petroleum hydrocarbon/ml of seawater/hour.

Recent studies have been performed in large (3'x20') seawater tanks filled with Narragansett Bay seawater. Three tanks were used with one tank serving as a control, a second dosed with Kuwait crude oil and the third tank contained Kuwait crude dispersed into the water column with Corexit 9527. Samples were removed at intervals to determine the number of bacteria (total heterotrophs) and rate of hexadecane utilization and naphthalene utilization as measured by mineralization to carbon dioxide using ¹⁴C labeled substrates (Table 3).

Table 3

Mineralization rates by Narragansett Bay
seawater populations at 18C

Test Day	No. Bacteria	Tank	Mineralization Rate ng/hr	
			n-hexadecane	naphthalene
0	1.6×10^4	Control	11	26
		Oil	11	14
		Dispersed		
		Oil	33	2
7	1.7×10^4	Control	1	12
		Oil	45	0.9
		Dispersed		
		Oil	36	4.0

It is evident that the degradation rates for representative hydrocarbons fall into the general range described by the BOD method. The bacterial content of this water is rather high compared to open ocean seawater, and in this test series was run at about the highest seasonal temperature for this body of water. It does appear that degradation rates on the order of a few ng of substrate per hour are more realistic than rates on the order of ug or even mg of substrate per hour or even per day.

3. Gaspee Point Sediment Study

Many of the techniques developed in the early stage of this project were used to investigate the response of sediment populations following a major spill of No. 6 fuel oil in northern Narragansett Bay. (Pierce, Cundell and Traxler, 1975) The study demonstrated a rapid enrichment in the sediment population for hydrocarbon degrading bacteria. The enrichment occurred by day 16 following the spill and a significant population of hydrocarbon-degrading bacteria was maintained in the beach sediment for at least one year. The concentration of petroleum hydrocarbons in the mid-tide sediments declined rapidly during the bacterial enrichment period, remained constant during the summer, then declined to a low concentration after one year. Hydrocarbon degradation was apparent during the winter months at a rate less than 1ug of hydrocarbon per gram of dry sediment per day.

A more detailed discussion of degradation rates, substrates utilized and the oil spill study can be found in the annual reports of this contract (Traxler and Cundell, 1975; Traxler and Cundell, 1976).

4. Inclusions of Hydrocarbon Grown Microorganisms

In the study of the various organisms used in this project it was observed that the cells grown on n-alkanes contained intracellular inclusions but the same organism grown on amino acids or other water soluble substrates did not contain the inclusions. This phenomenon has been observed by most investigators working with microorganisms which metabolize n-alkanes. Kennedy, et. al. (1975) suggest that the inclusions represent hydrocarbon pooled within the cell. This thesis does not seem reasonable when one considers the proposed location of the hydrocarbon oxygenases as associated with the cellular membrane, through which the hydrocarbon molecules would be actively transported.

The morphology and ultrastructure of a Penicillium sp. grown on n-hexadecane and on peptone were compared using transmission and scanning electron microscopy (Cundell, Mueller and Traxler, 1976). The fungus grew as hollow mycelial balls surrounding individual hydrocarbon droplets in liquid hexadecane medium and as solid mycelial balls in liquid peptone medium. A dense layer of fungal mycelium that showed irregular forms, fusion and increase in hyphal size was formed at the hydrocarbon-water interface. Inclusions were present in the hexadecane grown fungus but were absent when the fungus was grown on peptone. These observations complete the catalog of organism types in which the hydrocarbon derived inclusions have been demonstrated. The filamentous fungi such as this Penicillium present certain limitations for studies on the chemical nature of the inclusions. For example, extracellular hydrocarbon is trapped between filaments in the mycelial matt and is virtually impossible to wash free from the biological material.

A marine Arthrobacter isolated in the early phase of this project was chosen to continue our investigation of the intracellular inclusions of hydrocarbon origin. Thin sections of this Arthrobacter grown on n-hexadecane showed numerous lucent areas and electron dense areas surrounded by a lucent area (Figures 1,2). The number of lucent areas per cell varies and in many instances they occupy up to 40% of the cellular volume. Careful examination of the lucent areas with high magnification demonstrated no ultrastructural elements in the deposits. The cytoplasmic material intrudes into the deposits slightly and there is no evidence of a membrane separating the inclusion and the cytoplasm. In contrast the peptone grown cells (Figure 3) do not contain either of the inclusions found in the hydrocarbon grown cells.

A higher magnification of the electron dense structure (Figure 4) demonstrated considerable ultrastructural detail. This is not a membrane bound structure nor is it associated with the cytoplasmic membrane. The fine structural nature suggests it may be an "oxisome" which contains the enzymes involved in hydrocarbon oxidation. The dense structure is surrounded by a lucent area like the other inclusion. If indeed the electron dense structure is an oxisome and the site of enzyme activity, the lucent areas may represent oxidation products such as fatty acids. Normally, there are two of these dense structures per cell: some sections did not have inclusions which is related to the plane of the cut.

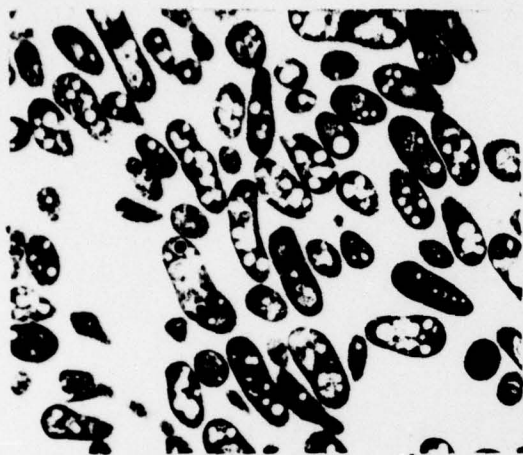


Fig. 1 Hexadecane grown
Arthrobacter. Thin section



Fig. 2. Hexadecane grown
Arthrobacter. (a) Luscent
structure (b) Dense structure



Fig. 3. Peptone grown
Arthrobacter. Thin section



Fig. 4. Hexadecane grown
Arthrobacter. Dense structure
high magnification

The electron microscope techniques have been valuable in that they identified the presence of the structures and provided a rough quantitation in relationship to cell volume. The basic issue of these deposits is related to their chemical nature. If they represent pooled hydrocarbon they become significant in food chain magnification. If, on the other hand, they represent oxygenated products such as fatty acids there is less ecological significance in relationship to the food chain. It is logical to assume that some small quantity of hydrocarbon will be located intracellularly in the actively growing cells. If pooling of hydrocarbon does occur the intracellular hydrocarbon will represent a major percentage of the cell dry weight.

Arthrobacter was grown in basal salts medium containing 0.4% hexadecane as the growth substrate for 4 days at 24C. The cells were harvested by collection on a Millipore membrane filter, then washed 8 times in 20 ml of n-dodecane. After washing the cells were resuspended in 20 ml of n-hexane and sonicated for 3 minutes, using 15 second sonication bursts with the suspension cooled in an ice water bath. The sonicate was centrifuges at 15,000 rpm for 10 minutes to remove cellular debris.

The final hexane extract was examined by quantitative gas chromatography and the amount of n-hexadecane present in the hexane extract of sonicated cells was calculated on the basis of 1 mg dry weight of the original cell suspension. The experiment has been replicated 6 times and n-hexadecane was not detected in 4 of the experiments, but was present in two experiments. The quantity of n-hexadecane present per mg dry weight of cells was 7.32 ug and 6.25 ug. This represents an average of 30 nmoles of n-hexadecane per mg of cells. On a percentage weight basis the total hexadecane content of the cells averaged 0.678% for the two experiments which detected the substrate hydrocarbon. Since estimates of the inclusion volume indicated they occupied up to 40% of the total cell volume it is reasonable to assume that the inclusions do not represent the pooled substrate n-hexadecane.

To verify this quantitation experiment, partition experiments were performed using substrate labeled with ^{14}C . The experimental protocol was identical to the quantitation experiments up to the hexane extract stage. The hexane sonicate, with debris removed, was partitioned against 100 ml of water (pH 9.0). At this pH any fatty acids present in the cells would be converted to the more soluble sodium salt whereas hexadecane would be most soluble in the hexane phase of the partition. As a control, ^{14}C -n-hexadecane dissolved in n-hexane was partitioned against water at pH 9.0. The results of these experiments are shown in Table 4.

Table 4
Partition Data From n-hexadecane grown cells

Phase	u Cu ^{14}C in Experiments			Hexadecane Control
	Expt. 1	Expt. 2	Expt. 3	
Hexane	0.08	0.04	0.01	0.94
Aqueous	0.16	0.164	0.04	0.21
Ratio	1/2.0	1/3.7	1/4.0	4.43/1.0



Fig. 5. Hexadecane grown cells,
acrylate inhibited

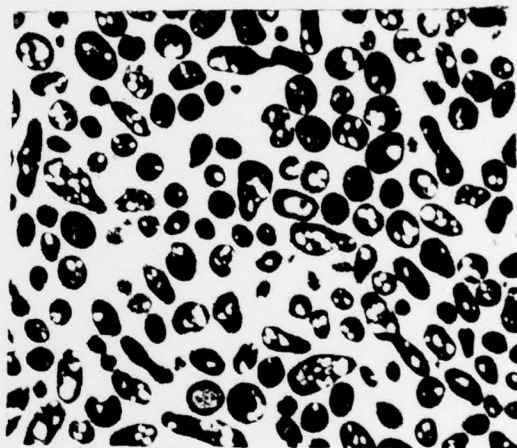


Fig. 6. Hexadecane grown cells,
no acrylate

There is a slight solubility of n-hexadecane in water so that a small quantity of hydrocarbon can be dissolved in water as evidenced by the hexadecane control experiment. It is also rather certain that some unconverted hexadecane will be present in actively growing cells. However, these partitions are greatly in favor of fatty acids being the major labeled component in the cells. These observations support the quantitation experiments which indicate that the deposit represent fatty acid or some other oxygenated product of hydrocarbon metabolism rather than pooled hydrocarbon.

A final experiment was performed which also supports the theory that the intracellular deposits are not pooled hydrocarbon. Two cultures of Arthrobacter were grown on n-hexadecane, one flask of which contained acrylate, an inhibitor of beta-oxidation. The cells were collected, and examined by transmission electron microscopy to determine the presence or absence of the inclusions. Figures 5 and 6 are sections examined from the two batches of cells. The acrylate inhibited cells contain no inclusions whereas the non-inhibited cells have abundant inclusions. Acrylate inhibition caused the formation of early fatty acid which is a repressor of the hydrocarbon oxygenases thus preventing hydrocarbon uptake. This is evidenced by lack of growth in the acrylate inhibited culture.

D. Conclusions:

The following principles and observations have emerged from this investigation:

1. A diverse population of hydrocarbon-utilizing bacteria are found in all sources sampled.
2. These bacteria degrade aliphatic, naphthenic (cyclic) and aromatic hydrocarbons.
3. There is a seasonal selection of hydrocarbon-utilizing bacteria.
4. Reduced isolation temperatures (below 15C) yield psychrotolerant organisms.
5. Reduced incubation temperatures result in increased lag phase and the generation time and the Q_{10} may vary from 1.3 to 2.4.
6. Rapid enrichment of hydrocarbon-utilizing organisms occurs in sediments in response to spilled fuel oil.
7. Marked reductions in hydrocarbon fractions of petroleum in the sediment coincided with the enrichment of these microorganisms in the sediments.

8. Degradation rates in situ are much lower than laboratory measured rates and fall into the order of magnitude of ng of substrate degraded per day rather than ug or mg as reported by other investigators.

9. Organisms growing on hydrocarbons form two types of intracellular inclusions not found in the same organism grown on water soluble substrates such as peptone.

10. These inclusions are not characterized as hydrocarbon but represent a hydrocarbon degradation product such as fatty acid.

E. Personnel contributing to this project:

1. Richard W. Traxler, Professor and Chairman, Department of Plant Pathology Entomology. Microbiologist and project leader.
2. Dr. Anthony M. Cundell, Research Associate. Microbiologist.
3. Dr. Richard H. Pierce, Jr., Research Associate. Chemist.
4. Mr. Robert Young, Undergraduate student, microbiologist.
5. Mr. Gerald Keeler, Undergraduate student, microbiologist.
6. Ms. Lauren Hall, Undergraduate student, microbiologist.
7. Dr. Gail Vander Molen, Research Associate. Electron Microscopist and Cell Biologist.
8. Dr. Walter C. Mueller, Professor of Plant Pathology. Ultrastructure.
9. Mr. William M. Griffin, Graduate Research Assistant, microbiologist.

F. Publications from this contract:

1. Cundell, A.M. and R.W. Traxler. 1974. Hydrocarbon-degrading bacteria associated with an arctic oil seep. *Devel. Indust. Microbiol.*, 15:250-255.
2. Pierce, R.H., A.M. Cundell, and R.W. Traxler. 1975. Persistence and degradation of spilled residual fuel oil on an estuarine beach. *Appl. Microbiol.*, 29:646-652.
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